Gangliogenesis in the Prosobranch Gastropod *Ilyanassa obsoleta*

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Abstract:

We determined that the larval nervous system of *Ilyanassa obsoleta* contains paired cerebral, pleural, pedal, buccal, and intestinal ganglia and unpaired apical, osphradial, and visceral ganglia. We used a modified form of NADPH diaphorase histochemistry to compare the neuroanatomy of precompetent (including specimens 6, 8, and 12 days after hatching), competent, and metamorphosing larvae with postmetamorphic juveniles. This method highlighted ganglionic neuropils and allowed us to identify individual ganglia at various stages of development, thereby laying a foundation for concurrent histochemical studies.

The first ganglia to form were the unpaired apical and osphradial ganglia and the paired cerebral and pedal ganglia. In larvae 6 days after hatching, the neuropil had already appeared in the apical and osphradial ganglia. Neuropil began to be apparent in the cerebral and pedal ganglia 2 days later. At that time, the pleural and buccal ganglia were identifiable and adjacent to the posterior edge of the cerebral ganglia. The ganglia of the visceral loop were concurrently recognizable, although the supraintestinal ganglion developed slightly earlier than the subintestinal and visceral ganglia. By 12 days after hatching, all of the major adult ganglia were discernible. The apical ganglion was retained by newly metamorphosed juveniles, but not by juveniles 2 days later. After metamorphosis was complete, the central nervous system (CNS) was consolidated into its juvenile form with ipsilateral cerebral and pleural ganglia being partially fused. The metamorphic translocation of ganglia, which included a caudal relocation of the cerebrals and the migration of the buccals from above the esophagus to a position below it, correlated with the movement of the proboscis to the dorsal part of the head.
Indexing terms: ganglion, metamorphosis, neuroanatomy, neuropil, veliger

Abbreviations

A	apical ganglion	P	pigment
A-P	anterior-posterior axis	PC	pedal commissure
BC	buccal commissure	PD	pedal ganglion
B	buccal ganglion	PP	propodial ganglion
C	cerebral ganglion	PL	pleural ganglion
CC	cerebral commissure	R	radula
E	eyespot	RB	right buccal ganglion
ES	esophagus	RC	right cerebral ganglion
F	foot	RG	right cerebropleural ganglion
G	gut	RP	right pleural ganglion
LB	left buccal ganglion	S	statocyst
LC	left cerebral ganglion	SB	subintestinal ganglion
LG	left cerebropleural ganglion	SP	supraintestinal ganglion
LP	left pleural ganglion	V	velum
O	osphradial ganglion	VI	visceral ganglion
OP	operculum		

Article:

In evolutionary terms, gangliogenesis in gastropod molluscs is highly plastic, with even closely related species showing sigmificant differences in their embryological and larval development (D'Asaro, 1969). In the caenogastropod prosobranchs (formerly the meso- and neo-gastropods; Cox, 1960; Ponder and Waren, 1988), embryological and larval development is further complicated by a tendency toward the replacement of a long-lived planktonic veliger stage by intracapsular development, culminating in the release of a crawling juvenile

(Thorson, 1950; Fretter and Graham, 1962). There are general patterns in the developmental appearance of the ganglia of the CNS (extensive reviews in Fretter and Graham, 1962; Raven, 1966; Hyman, 1967), as will be briefly discussed in a separate section below, but numerous differences occur, especially during the ontogeny of the ganglia associated with the visceral loop and osphradium and in the amount of ganglionic fusion in the more derived caenogastropods (Bouvier, 1887). This variability makes it difficult to undertake neuroanatomical studies relating to larval ganglionic functions in any particular species without documenting its developmental pattern. The marine mud snail *Ilyanassa obsoleta* and its congeneric relatives have been used extensively for studies of molluscan development (reviews in Raven, 1966; Hyman, 1967) and larval life histories (Scheltema, 1961, 1962; Fretter and Graham, 1962) and the configuration of the adult nervous system is known for congeners of this species (Bouvier, 1887; Fretter and Graham, 1962). Previously, little was known about gangliogenesis in this genus and published reports of gangliogenesis in other prosobranchs, such as *Littorina obtusata* (Delsman, 1914), *Thais haemastoma floridana* (D'Asaro, 1966), or *Marisa cornuarietis* (Demian and Yousif, 1975), concern species in different families and even different orders Similarly, most neuroanatomical studies on newly metamorphosed gastropods have been conducted on opisthobranchs (Kempf et al., 1987; Page, 1992a,b; Carroll and Kempf, 1994), whose nervous systems can show anatomical arrangements that are significantly different from those of the caenogastropods (Bullock, 1965).

To better understand the neuroanatomical changes that occur before, during, and just after metamorphosis in *Ilyanassa obsoleta*, we compared the developing nervous systems of larvae and young juveniles to those in metamorphosing larvae. Our studies allowed us to describe changes in ganglionic organization that occur along the visceral loop, the connectives that pass above and below the digestive tract (Bouvier, 1887; Bullock, 1965) and changes that occur during the loss of the apical ganglion. This structure has recently been identified in only a few larval gastropods (Marois et al., 1993; Lin and Leise, 1994; Leise, 1996) and is more substantial than the previously described apical or cephalic sensory organ (Conklin, 1897; Bonar, 1978a,b).

MATERIALS AND METHODS

Egg capsules were collected twice per week from laboratory populations of adult *Ilyanassa obsoleta* (Say, 1922). Hatched larvae were raised at room temperature in an aerated culture system in a 1:1 solution of 0.2 μm filtered natural seawater (FSW) and instant ocean (FIO) containing 50 μg/ml each of penicillin G and streptomycin sulfate (Miller and Hadfield, 1986). Larvae were fed a combination of the algae *Isochrysis galbana, Nannochloropsis sp.*, and *Monocrypsis sp.* that were also cultured in the laboratory. Larvae became competent to metamorphose about 3 weeks after hatching.

Specimen preparation

Five different groups of *Ilyanassa* larvae were examined, including individuals at 6 days (325-450 μm in shell length, n=10), 8 days (~500 μm, n=10), and 12 days (~600 μm, n=10) after hatching, competent (> 600 μm, n=15), and metamorphosing larvae (n=8) (Figs. 1, 2). We also studied two groups of juveniles. Embryos and newly hatched larvae were not examined because of difficulty in decalcifying them. Larvae at 8 and 12 days after hatching were chosen because they displayed significant neuroanatomical differences from younger and older larvae. Competent larvae are about 600 μm in shell length (Scheltema, 1962) and over 80% metamorphose within 48 hours in response to 10^{-4} M serotonin (5-HT; Levantine and Bonar, 1986). The initiation of larval metamorphosis after exposure to a natural or an artificial inducer can vary temporally from a few minutes to many hours, but once induced, metamorphosis to a young juvenile stage (including complete loss of the velum) required approximately 1 day.

Other animals were staged by external morphological criteria: metamorphosing larvae bear a pair of small mounds of tissue that are the unciliated remnants of the velum (Fig. 2; Pires and Hadfield, 1991). Newly metamorphosed juveniles (approximately 2 days after induction, n=5) had no such mounds of tissue and their proboscides were not yet fully developed. Advanced juveniles (n=15) were obtained by inducing metamorphosis with 10^{-4} M 5-HT for 2 days and culturing them in a 1:1 solution of FSW and FIO for an additional 2 days. Occasionally, animals morphologically similar to advanced juveniles were obtained from cultures with spontaneously metamorphosed larvae. All animals were decalcified in Ca^{2+} free, pH 6.8-7.0

seawater over-night (Pires and Hadfield, 1993) followed by three rinses in FIO. Before fixation, all specimens were anesthetized in ice-cold FIO.

NADPH-diaphorase histochemistry

The application of NADPH-diaphorase (NADPHd) histochemistry to neurons containing the enzyme nitric oxide synthase results in a blue formazan product (Hope and Vincent, 1989; Elofsson et al., 1993). In our early experiments, light blue, nonspecific staining highlighted all of the ganglionic neuropils, while NADPHd-positive activity occurred as dark punctae scattered within the neuropils. The nonspecific NADPHd staining aided our identification of newly differentiating ganglia at different developmental stages. Anesthetized larvae were fixed at 4°C for 1.5 hours in chilled 4% paraformaldehyde in a 0.1 M phosphate buffer solution (PBS) at pH 7.6 and then rinsed once with 0.5 M PBS (Elofsson et al., 1993; K. Lukowiak, personal communication). After being rinsed with PBS, animals were immersed in the dark at room temperature for 3 hours in the incubation solution, which contained 0.5 mM Nitroblue tetrazolium (NBT), 0.1 mM dicumoral, 0.25% Triton X-100, and 1 mM β-NADPH in a 0.5 M Tris-HC1 buffer solution (TBS) at pH 8.0. The incubation solution was freshly prepared 20-30 minutes before use.

After incubation, specimens were rinsed in TBS three times, dehydrated in ethanol, and then embedded within 2 days of fixation. Tissues were infiltrated with 1:1 and 1:2 mixtures of absolute ethanol and soft Spurr's resin for 45 minutes each, followed by two changes of pure Spurr's resin for 1.5 hours (Spurr, 1969). Specimens in pure Spurr's resin were polymerized in a 60°C oven for 16 hours. Serial transverse and sagittal sections were cut at 8 μm with a Reichert-Jung 2040 microtome. Sections were examined and photographed on an Olympus BH2 compound microscope equipped with Differential Interference Contrast optics. Photographs of ganglionic neuropils expressing NADPHd activity will be discussed in the following paper (Lin and Leise, 1996).

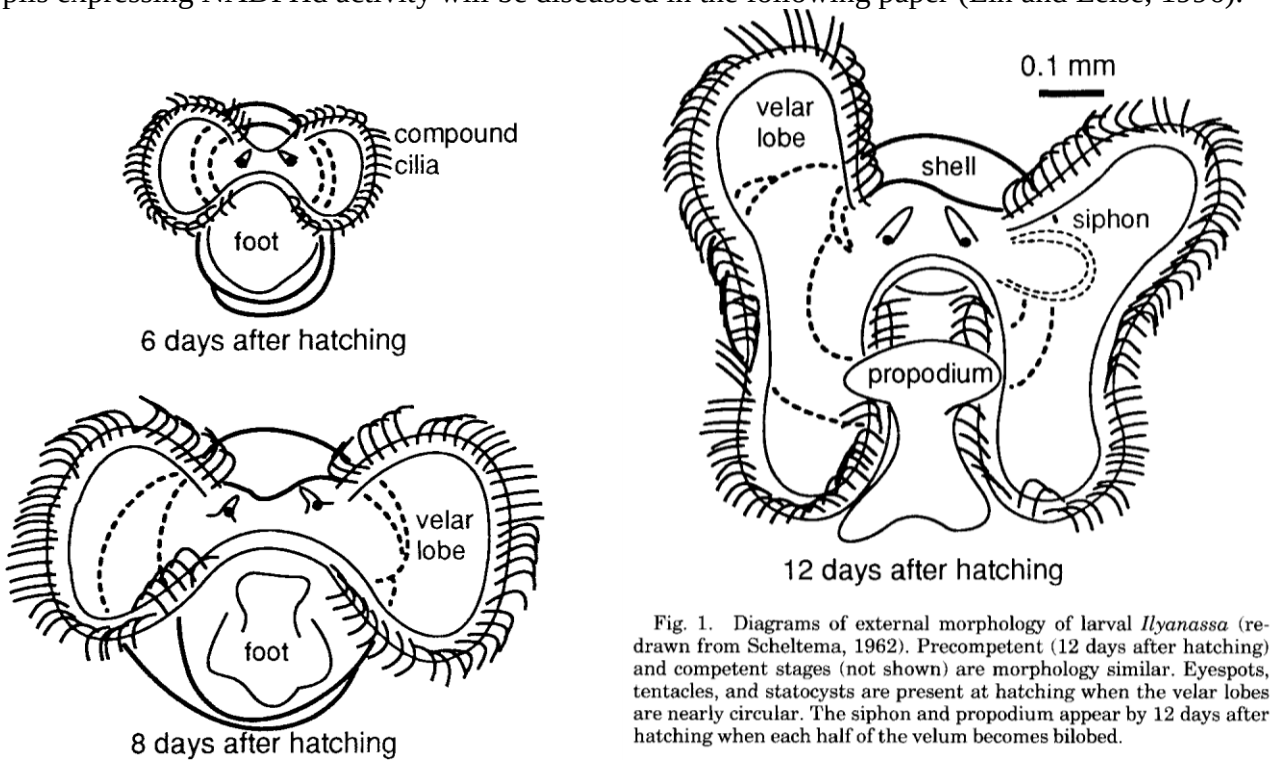

Fig. 1. Diagrams of external morphology of larval *Ilyanassa* (redrawn from Scheltema, 1962). Precompetent (12 days after hatching) and competent stages (not shown) are morphology similar. Eyespots, tentacles, and statocysts are present at hatching when the velar lobes are nearly circular. The siphon and propodium appear by 12 days after hatching when each half of the velum becomes bilobed.

REVIEW OF GANGLIOGENESIS

Ganglia of the gastropod central nervous system (CNS) develop from ectodermal placodes throughout the embryonic and larval stages. Cells from these placodes ingress and actively divide to become recognizable as the major ganglia in swimming larvae (reviewed in Raven, 1966; Hyman, 1967). The cerebral ganglia, lying on each dorsolateral side of the head and connected by a broad commissure, are reportedly the first pair formed and are evident at hatching. These ganglia send connectives to the buccal, pleural, and pedal ganglia and innervate muscles and sensory regions of the head, including the apical sensory organ, the velar lobes, eyes, statocysts,

tentacles, and skin (Smith, 1935; Crofts, 1938; Fretter and Graham, 1962; Bullock, 1965; D'Asaro, 1965, 1966, 1969; Bedford, 1966; Raven, 1966; Hyman, 1967; Demian and Yousif, 1975; Voltzow, 1994).

The pedal ganglia, also evident at hatching, are connected by one or more commissures or may directly contact each other or the developing cerebral ganglia. The pedal ganglia are situated below the developing buccal mass in the more proximal portion of the larval foot. The pedals innervate the foot and its derivatives and are linked to the pleural ganglia by connectives (Smith, 1935; Crofts, 1938; Bullock, 1965; D'Asaro, 1965; Hyman, 1967). Towards the end of the larval phase, the foot differentiates into two major regions: an anterior propodium, which gives the foot a crawling surface (Page, 1994), and the more posterior metapodium, which bears the operculum. The pedal ganglia send large nerves into both of these regions (Moritz, 1939; D'Asaro, 1965; Hyman, 1967).

In prosobranchs with encapsulated development, the pleural ganglia may or may not be evident at hatching when studied by light microscopy (LM; Crofts, 1938; Moritz, 1939; D'Asaro, 1965, 1966, 1969; Bedford, 1966; Demian and Yousif, 1975). In general, the pleural ganglia develop dorsally to the statocysts and posterodorsally to the cerebral ganglia (Smith, 1935; Crofts, 1938; Raven, 1966). The pleural ganglia are often adjacent to or fused with the pedals in archaeogastropods (Smith, 1935; Crofts, 1938; Fretter and Graham, 1962; Raven, 1966; Hyman, 1967), but in more derived prosobranchs the pleurals lie closer to the cerebral ganglia (D'Asaro, 1965; Raven, 1966; Hasz-pruner, 1993). This may be true in some opisthobranchs (Thompson, 1958; Bickell and Chia, 1979; Bickell and Kempf, 1983; Page, 1992b), although there is still some controversy over the homology between prosobranch and opisthobranch pleural ganglia (Page, 1994; Carroll and Kempf, 1994). The pleural ganglia arise as posterior portions of the cerebral ganglia in the prosobranch *Littorina obtusata* (Delsman, 1914) and the opisthobranchs *Tritonia hombergi* (Thompson, 1962) and *Aeolidiella alderi* (Tardy, 1970); a constriction divides each cerebral ganglionic anlage into two regions during gangliogenesis (reviewed in Raven, 1966). In the prosobranchs, the pleural ganglia innervate the mantle and columellar muscles (Hyman, 1967).

The paired buccal ganglia are usually connected by a single commissure and develop from the wall of the foregut (D'Asaro, 1965; Raven, 1966; Hyman, 1967; Voltzow, 1994). The ontogeny of the buccals varies between species and has been less well studied than that of the above ganglia. The buccals may arise embryologically, before hatching (Bed-ford, 1966; Demian and Yousif, 1975) or after hatching (D'Asaro, 1969). The buccal ganglia can also appear midway through larval development, as they do in the limpet *Patella vulgata* (Smith, 1935) and the queen conch *Strom-bus gigas* (D'Asaro, 1965). In adults, these ganglia inner-vate the walls of the pharynx, the muscles of the buccal mass, the foregut glands, radular apparatus, stomach, and occasionally other viscera (Bullock, 1965; Hyman, 1967; Voltzow, 1994).

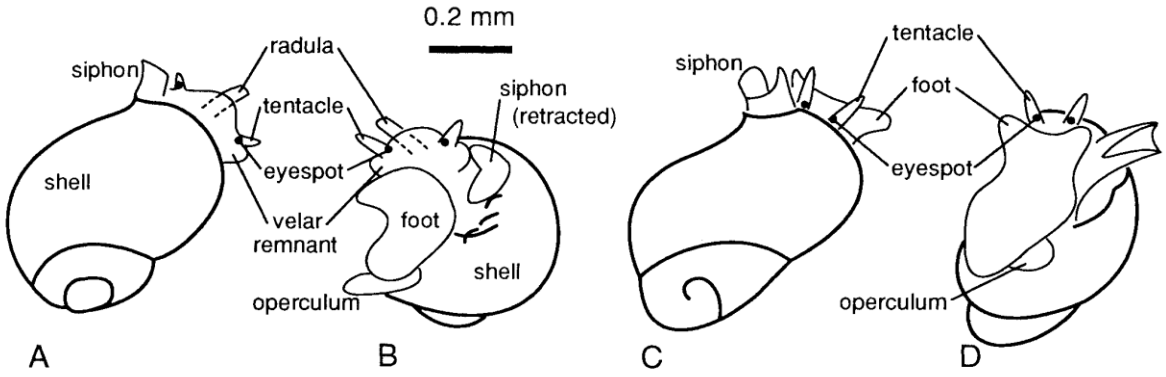

Fig. 2. Diagrams of dorsal, A,C, and ventral B,D, views of the external morphology of metamorphosing larvae and juveniles showing changes in their heads. **A,B:** Metamorphosing larvae have exposed radulae and velar remnants that lack cilia. **C,D:** The distance between the eyes in these juveniles is less than in competent or metamorphosing larvae.

The ontogeny of the remaining ganglia, the intestinals, visceral, and osphradial ganglia, has also been neglected in comparison to that of the more anterior ganglia. The intestinal ganglia, also known as parietal or esophageal ganglia, generally appear by the middle or the end of the larval life (Smith, 1935; D'Asaro, 1965, 1966, 1969; Demian and Yousif, 1975). The intestinal ganglia lie along the visceral loop, the connectives that emerge from the pleural ganglia and pass above and below the digestive tract (D'Asaro, 1965; Raven, 1966; Hyman, 1967;

Dorsett, 1986; Voltzow, 1994). These ganglia and connectives are asymmetrical and can vary widely in position because of the torsion-induced streptonery (crossing of the nerve cords) that takes place during the early veliger stage (Fretter and Graham, 1962; Raven, 1966; Hyman, 1967). Fusion of the intestinal ganglia with their pretorsional ipsilateral pleuropedal ganglion is reported for the caenogastropods *Marisa cornuarietis* (Demian and Yousif, 1975), *Crepidula adunca* (Moritz, 1939), and *Ampullaris sp.* (Honegger, 1974), such that the subintestinal ganglion is fused with the right pleural ganglion while the supraintestinal ganglion is displaced towards the left pleural. The intestinal ganglia innervate the kidney, gill, and osphradium, although this latter organ often contains a separate ganglion (D'Asaro, 1965; Hyman, 1967).

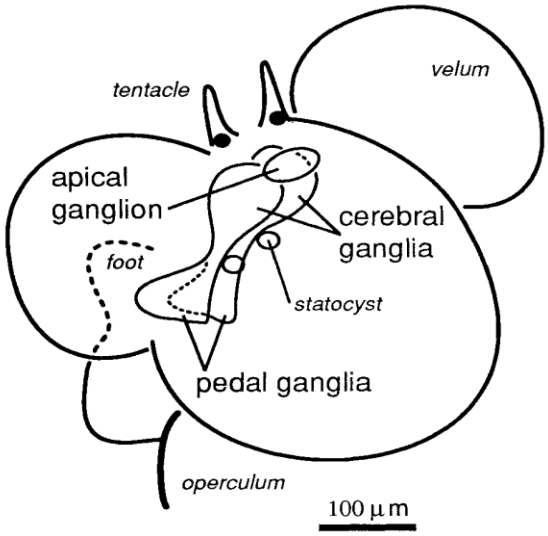

Fig. 3. Diagram of a veliger larva 6 days after hatching showing the spatial relationship of the ganglia of the central nervous system (CNS). The apical, cerebral, and pedal ganglia are present. The apical ganglion is adjacent to the cerebral commissure, anterodorsal to the statocysts. The osphradial ganglion, part of the peripheral nervous system, is on the left side of the animal but is excluded to maintain diagrammatic simplicity.

From each intestinal ganglion a connective proceeds posteriorly and contralaterally to join a visceral ganglion located at the beginning of the visceral mass (Raven, 1966; Hyman, 1967). This ganglion arises from an unpaired ectodermal thickening at the posterior end of the mantle cavity late in the veliger phase (Smith, 1935; D'Asaro 1965, 1966, 1969). The visceral ganglion innervates the caudal region of the gut, the anus, adjacent parts of the skin and body wall, reproductive organs, kidney, liver, and heart (Bullock, 1965; Hyman, 1967).

The chemosensory osphradium lies within the mantle cavity of gastropod molluscs (Hyman, 1967; Haszprunar, 1985; Voltzow, 1994) and in prosobranchs, its central, interior portion often contains a ganglion. Either this ganglion or the sensory neurons directly are connected to the posterior end of the supraintestinal ganglion by the osphradial nerve (Hyman, 1967). The ontogeny of this ganglion, like that of the buccals, intestinals, and visceral, is also not well known (D'Asaro, 1965, 1966, 1969).

RESULTS

Changes in the external morphology during larval growth of *Ilyanassa obsoleta* have been studied previously (Scheltema, 1961, 1962) and are illustrated diagrammatically in Figure 1. Upon induction of metamorphosis with 10^{-4} M 5-HT (Levantine and Bonar, 1986), the ciliated cells of the velum are shed, followed by a dramatic change in the configuration of the head. The remnants of the velar lobes decreased in size (Fig. 2) as did the distance between the eyes. However, only advanced juveniles (4 days after metamorphic induction) were able to rasp with their proboscides and ingest food. The only food items we observed them ingest were diatoms.

By 6 days after hatching, the larval nervous system consisted of the unpaired apical and osphradial ganglia, each with a well-developed neuropil, and the paired cerebral and pedal ganglia which, by LM, lacked neuropils (Figs. 3, 4). The apical ganglion lay above the developing cerebral commissure and was composed of about eight neurons and an internal neuropil. The cerebral commissure was continuous with the neuropil of the apical ganglion. The boundary between the cerebral and the pedal ganglia was indistinct.

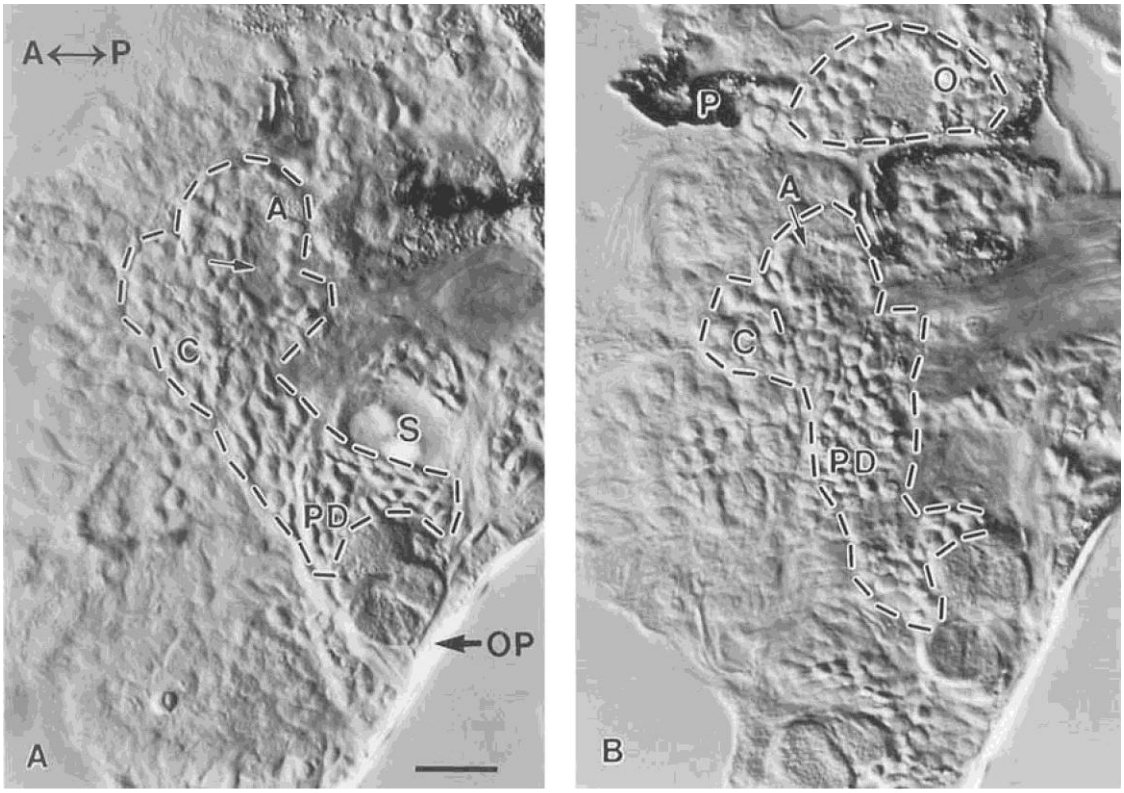

Fig. 4. Sagittal sections through larvae 6 days after hatching. **A:** Medial section. Apical and pedal ganglia are continuous with the cerebral ganglion. Of these ganglia, only the apical ganglion contains a neuropil (arrow). **B:** More lateral section includes the osphradial ganglion which also contains a central neuropil. Dashed lines show approximate extent of ganglia. Scale bar = 20 μm.

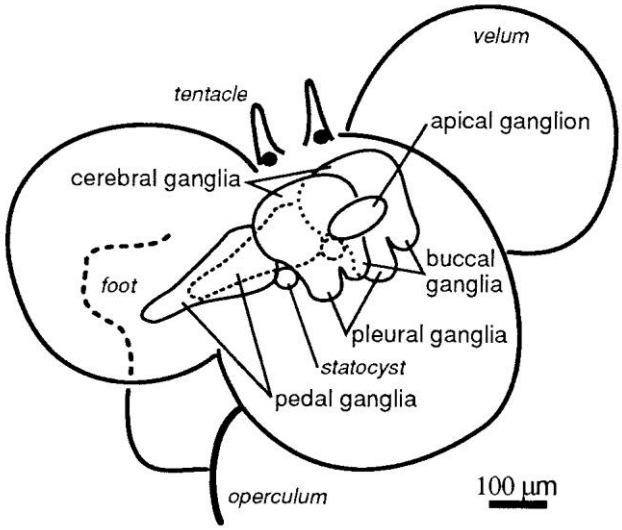

Fig. 5. Diagram of a veliger larva 8 days after hatching showing the spatial relationship of the CNS ganglia. The buccal and pleural ganglia now appear at the posterior portion of the cerebral ganglia. The apical ganglion retains its original position. The intestinal ganglia are also developing at the rear of the ganglionic mass but are not shown. The osphradial ganglion is again not drawn to maintain diagrammatic simplicity.

By 8 days after hatching, *Ilyanassa* larvae had well-developed cerebral, pedal, pleural, buccal, and apical ganglia (Figs. 5, 6). The supraintestinal and subintestinal ganglia were just developing at the rear of the ganglionic mass (not shown in Fig. 5). The cerebral and pedal ganglia consisted of cortices of neuronal somata surrounding central neuropils (Fig. 6A). The pedals, located ventrally to the statocysts, were linked by a single commissure. The osphradial ganglion was also larger and could easily be found on the left side of the animal (Fig. 6C). The apical ganglion was likewise larger than before and the number of its large neurons had increased to nearly a dozen. The pleural and the buccal ganglia started to appear at this stage and remained continuous with the cerebrals (Fig. 6A). The anlagen of the pleural ganglia lay posterior to those of the cerebrals and

posterodorsal to the statocysts. Connectives linking the pleural to the pedal ganglia were not yet clearly discernible. A short nerve (Fig. 6C) linked the osphradial ganglion to the supraintestinal ganglion, which lay below the inferior border of the entrance to the mantle cavity and above the esophagus. The intestinal ganglia are illustrated in later stages.

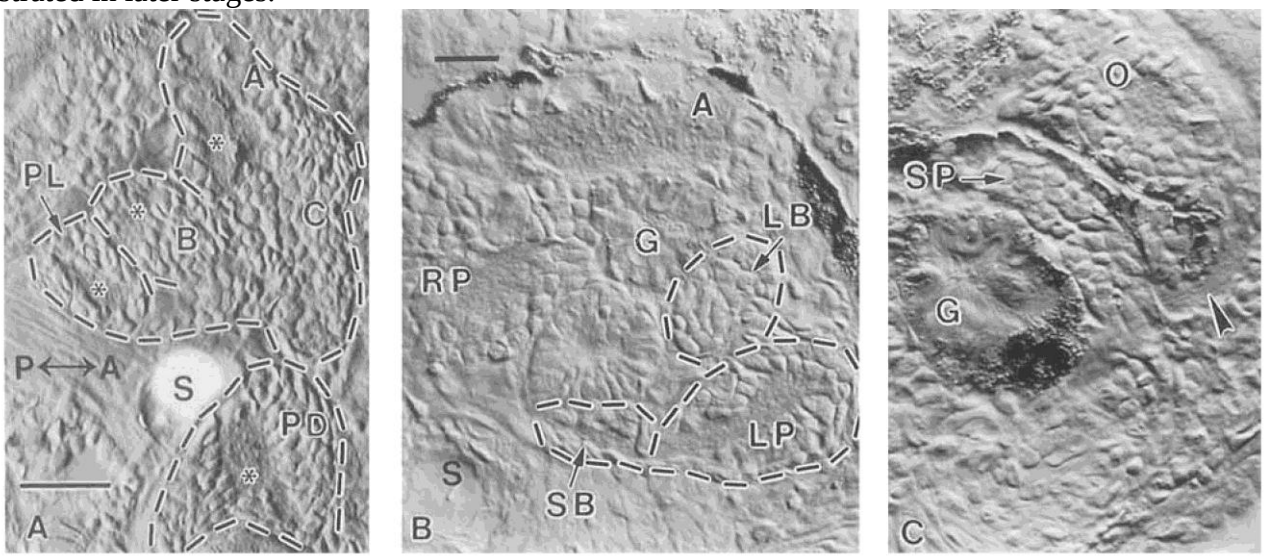

Fig. 6. **A:** Sagittal section through a larva 8 days after hatching. Neuropils (asterisks) in the cerebral, buccal, pleural, and pedal ganglia are now evident. The buccal and pleural ganglia arise at the posterior portion of the cerebral ganglia. B,C: Same age, transverse sections. **B:** Note asymmetry of pleural ganglia. **C:** A nerve (arrowhead in C) from the supraintestinal ganglion innervates the osphradium located on the animal's left side. Scale bars = 50 μm in A, 20 μm in B.

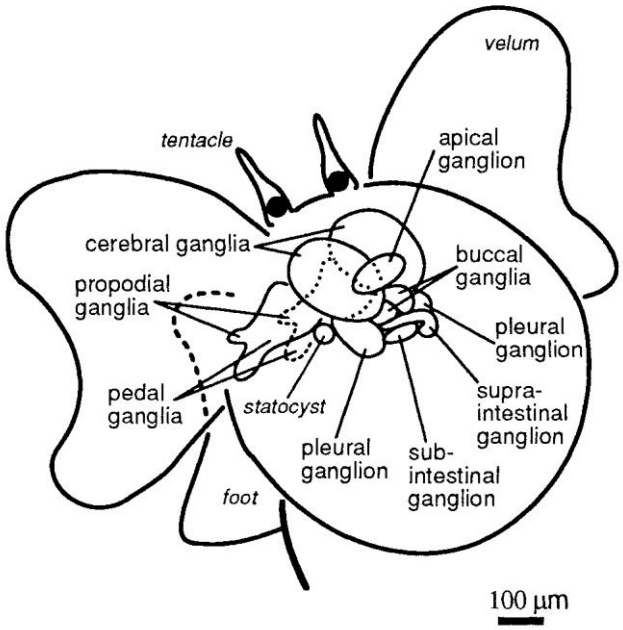

Fig. 7. Diagram of the CNS in a competent larva. The supraintestinal and subintestinal ganglia are individually identifiable and the buccal ganglia are now medial and mostly posterior to the cerebrals. Propodial ganglia have differentiated at the anteroventral ends of the pedal ganglia.

The nervous systems of precompetent larvae at 12 days after hatching were similar to those of competent larvae but had significant functional differences. Precompetent larvae could not be induced to metamorphose by exposure to a natural inducer and responded weakly and with a long latency to 10^{-4} M serotonin (unpublished data). Growth slowed at this stage with larvae showing only a small increase in size as they approached competence.

In a precompetent larva, the apical ganglion had enlarged and remained similarly sized through competence (Figs. 7-9). This ganglion contained 14-18 large neurons and did so until metamorphosis. The pleural ganglia

remained connected to the posterior portions of the cerebral ganglia and to their ipsilateral pedal ganglia by two connectives in competent larvae (Fig. 9B).

In precompetent and competent larvae, the paired buccal ganglia lay on either side of the foregut, partially posterior and medial to the cerebral ganglia (Figs. 8A,C, 9B,D). In transverse sections, the buccal ganglia and their commissure formed an asymmetrical dumbbell-shaped complex (Fig. 9D), with the right buccal ganglion tilted dorsally. The position of the pleurals was similarly canted (Figs. 8B—D, 9D,E). The body of the supraintestinal ganglion, which was linked by a nerve to the osphradial ganglion, began next to the left buccal ganglion, touched the left pleural ganglion and passed anteriorly and to the right to join the right pleural ganglion (Figs. 8C,D, 9E,F). The subintestinal ganglion was ventral to the esophagus (Figs. 8C,D, 9E) and its neuropil was connected to those of both pleural ganglia (Figs. 8C, 9E).

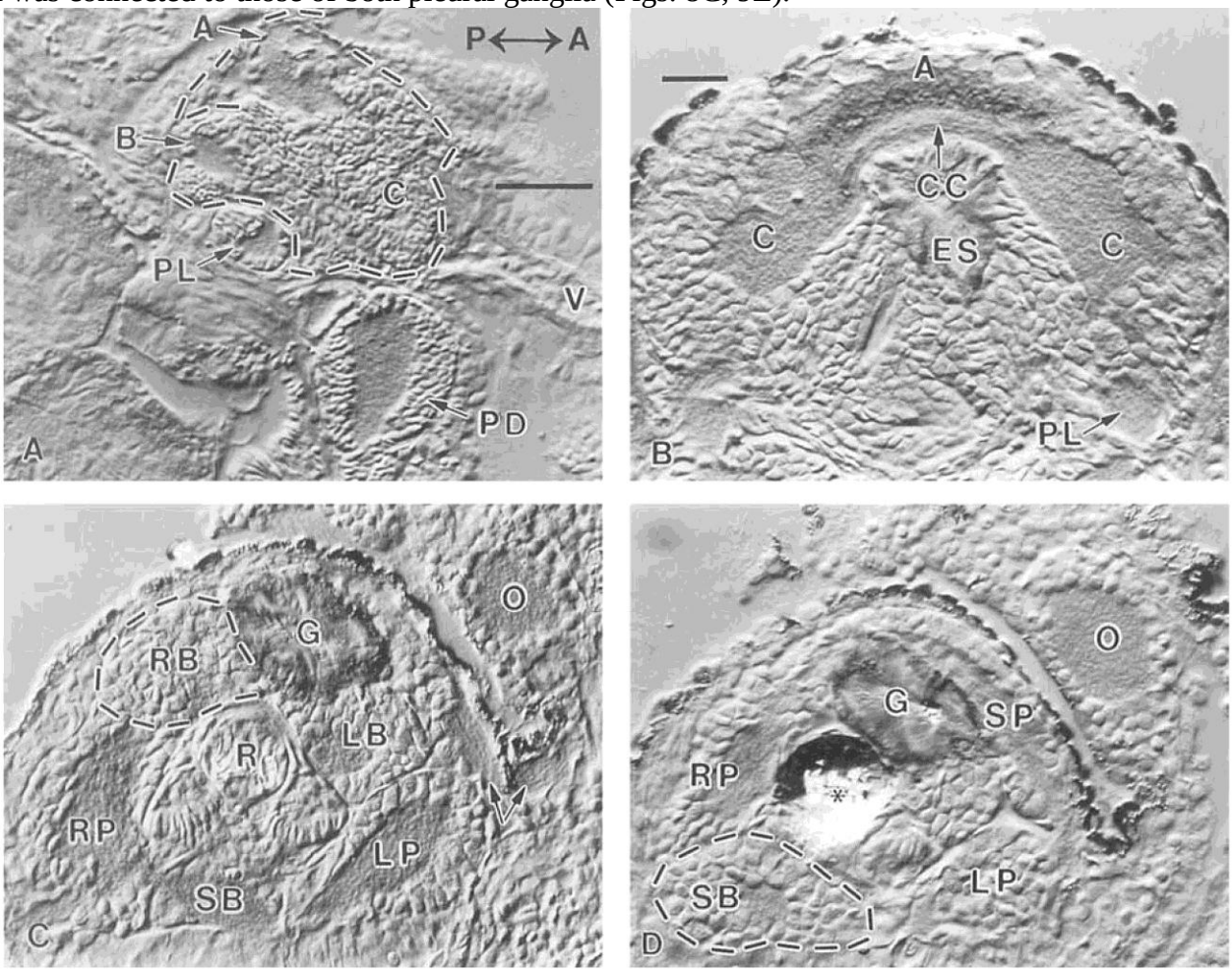

Fig. 8. Sagittal (A) and transverse (B,C,D) sections through a larva 12 days after hatching. **A:** Lateral view of the cerebral, buccal, pleural, and pedal ganglia. **B:** Pleural neuropils are separate from cerebral neuropils anterior to the statocysts. **C:** Note the partial fusion of the subintestinal ganglion with the right pleural ganglion and the nerve (arrows) joining the supraintestinal and osphradial ganglia. **D:** More posteriorly, the body of the supraintestinal ganglion curves above the gut and the neuropils of the subintestinal and right pleural ganglia are separate. Asterisk indicates sectioning artifact. Scale bars = 50 μm in A, 20 μm in B.

By competence, a pair of propodial ganglia had arisen from the anteroventral surface of the pedals and was joined to them by short connectives (Fig. 9A). The visceral ganglion appeared next to the posteromedial portion of the gut in competent larvae (Fig. 9F).

Two of the most obvious changes that occurred in the nervous systems of metamorphosing larvae were the decrease in size of the neuropil of the apical ganglion (cf. Figs. 8B, 10A) and the rearrangement of the its neurons before the larva completely lost its velum (Figs. 10, 11). The neurons clustered into 3-4 layers, an increase from the 1-2 layers generally seen before and during competence (Figs. 8B, 9A, 11). This reorganized ganglion made a small protrusion at the middle of the head (Fig. 10). Most of the other ganglia retained their same relative

positions. The visceral ganglion became fused with the subintestinal ganglion during metamorphosis (data not shown).

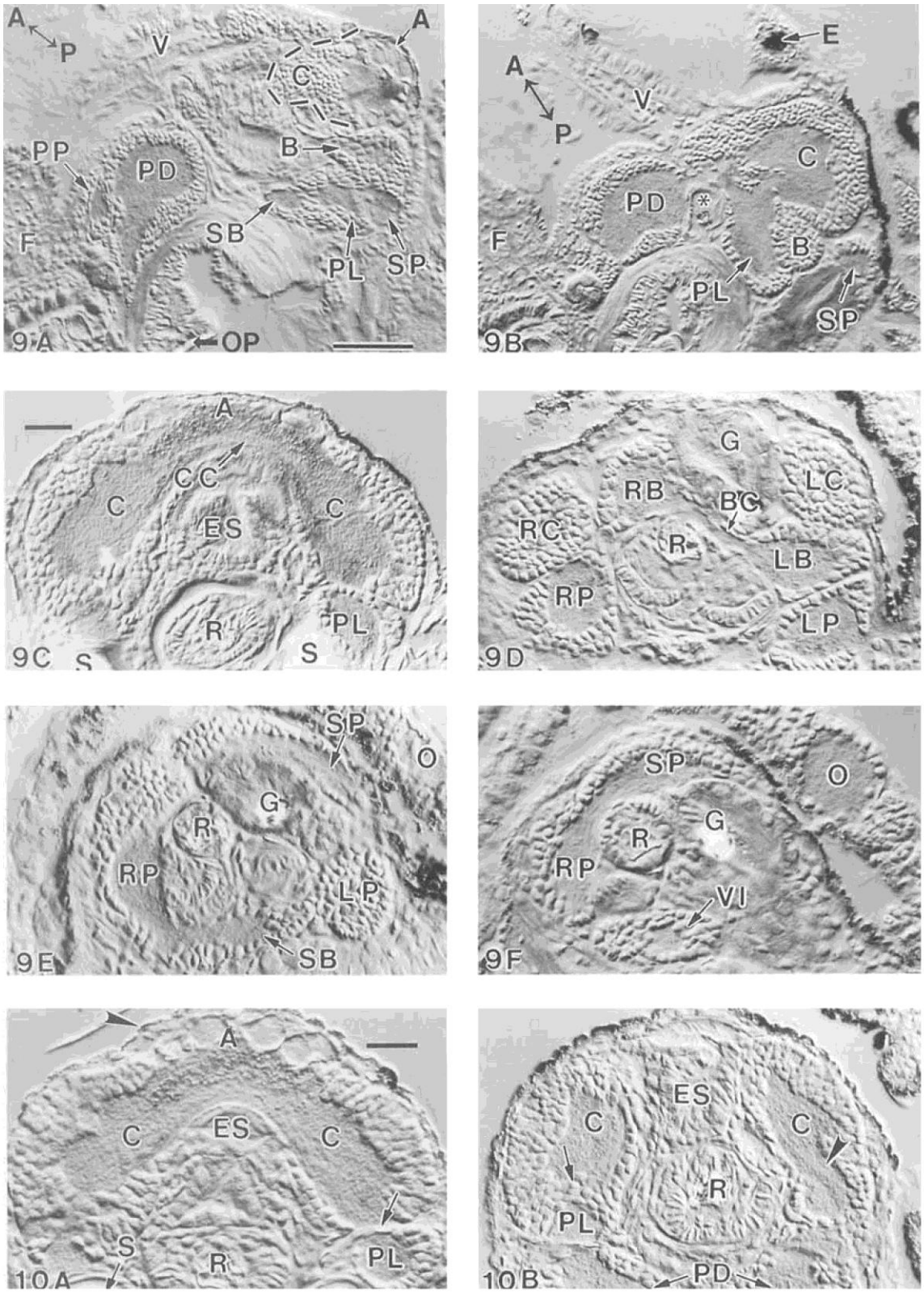

Fig. 9. Metamorphically competent larvae. **A:** Parasagittal section showing propodial ganglion located anterior to the base of the pedal ganglion. **B:** More lateral section showing enlarged neuropils in the cerebral, pedal, and pleural ganglia and their close interconnections. asterisk, statocyst. **C–E:** Sequential transverse sections. Note the asymmetrical positions of the pleural and buccal ganglia in comparison to those of the cerebral ganglia. D: The buccal ganglia are medial to the cerebrals and connected by a single commissure. E: The subintestinal ganglion curves below the foregut and is connected to the right pleural ganglion. **F:** Note the fusion of the supraintestinal and right pleural ganglia. The visceral ganglion is now apparent below the supraintestinal ganglion. Scale bars = 50 μm in A, 20 μm in C.

Fig. 10. Transverse sections through metamorphosing larvae. **A:** The apical ganglionic neuropil has decreased in its lateral extent and the neuropils of the pleural ganglia are separated from those of the cerebral ganglia by a septum (unlabelled arrow), or few cell layers (unlabelled arrow in B). The apical ganglion makes a slight protrusion at the top of the head (arrowhead). **B:** The neuropils of the cerebrals and pleurals are fused posterior to the statocysts (arrowhead). Scale bar = 20 μm.

After the disappearance of the velar remnants, the apical and buccal ganglia underwent significant changes in their positions. All newly metamorphosed juveniles retained an apical ganglion but it had moved caudally along with the cerebral commissure (Fig. 12A—C). The cerebral ganglia shifted in a ventrocaudal direction until they lay behind the pedal ganglia (cf. Figs. 9A,B, 12A). The buccal ganglia were now situated just below the cerebral commissure and the esophagus (Figs. 12A—C). The pleural ganglia remained behind the cerebrals and there was little change with respect to their original position (Fig. 12B,C). The positions of the supra- and sub-intestinal ganglia were likewise unchanged (Fig. 12C).

Each advanced juvenile had a conspicuous proboscis and some of their ganglia were in significantly different positions from those in competent larvae (cf. Figs. 7, 13, 14). The ganglia were relatively more consolidated with shorter connectives and commissures. All of the ganglia of the circumesophageal ring lay below the buccal mass (Fig. 15A, cerebrals not shown). The pleurals were fused with the cerebrals and the ganglia of the visceral loop (Figs. 13, 15C, 16). The neuropils and neurons of the apical ganglion had disappeared. The short cerebral commissure, which lay above the gut and relatively caudally, close to the fusion point of the visceral and subintestinal ganglia, brought the caudal regions of the cerebral ganglia into contact. The cerebral ganglia had migrated caudally to become located behind the pedals (Fig. 13). No connectives between the cerebral and the pedal ganglia were visible by LM. The buccals were now about twice the size of those in competent larvae (cf Figs. 9A,D, 15A,B). These enlarged ganglia were located between the cerebrals, anteromedially to the stato-cysts, and dorsal to the pedals (Figs. 13, 14). The buccal commissure was not visible by LM. In front of the stato-cysts, a thin septum separated the neuropils of the cerebral and the pleural ganglia (Fig. 15B) but behind the statocysts they were fused (Fig. 15C). In the left side of the body, the cerebropleural ganglion touched the visceral ganglion, but no fusion of neuropils occurred (Fig. 16A). The supraintestinal ganglion lay just caudally to the repositioned cerebral commissure (Fig. 16C). More posteriorly, the neuropils of the subintestinal and visceral ganglia were fused (Fig. 16D,E) as were those of the subintestinal and supraintestinal 'ganglia (Fig. 16F). No fusion occurred between the visceral and supraintestinal neuropils (Fig. 16F).

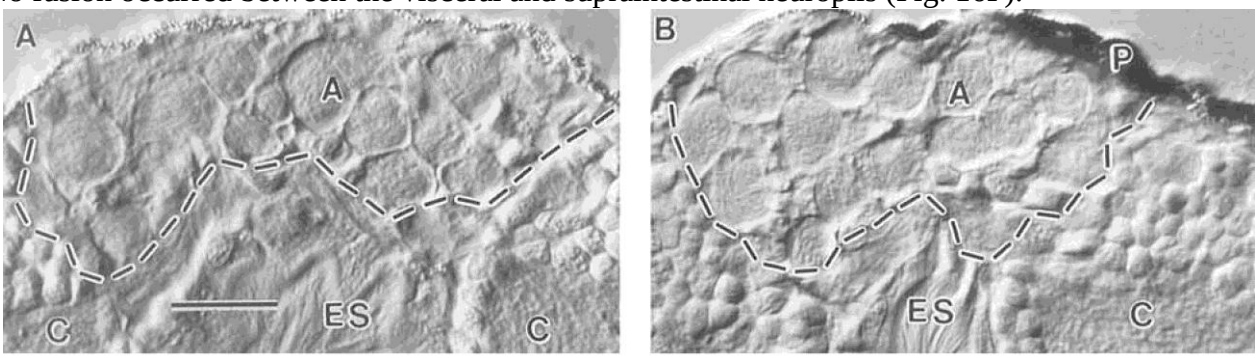

Fig. 11. Transverse sections through the anterior portion of the apical ganglion showing the rearrangement of the large neurons during metamorphosis. **A:** Before metamorphosis. **B:** During metamorphosis. Lines designate approximate extent of the apical ganglion. Scale bar = 20 μm.

DISCUSSION

In *Ilyanassa obsoleta,* all of the major ganglia had formed by 8 days after hatching except for the visceral ganglion (Table 1). As in other prosobranchs (Smith, 1935; Crofts, 1938; Moritz, 1939; D'Asaro, 1965, 1966, 1969; Raven, 1966; Demian and Yousif, 1975; Haszprunar, 1993), the cerebral and pedal ganglia of *Ilyanassa obsoleta* arose early in gangliogenesis, but in this species appeared to be preceded by the apical and osphradial ganglia which already contained central neuropils. The pleural and buccal ganglia developed shortly thereafter from posterior portions of the cerebral ganglia. The cerebrals and pleurals remained fused during larval and early juvenile development as they do in *Littorina obtusata* (Delsman, 1914; Raven, 1966). The pleural and cerebral ganglia occur as separate, but closely apposed ganglia in adult *Ilyanassa reticulata* (Fretter and Graham, 1962) and so in *I. obsoleta* are likely to separate during juvenile maturation. Thus, each cerebral anlage may actually give rise to three separate ganglia, a cerebral, pleural, and buccal ganglion. Similar origins for these ganglia are reported for other gastropods (Moritz, 1938; Thompson, 1962; Bedford, 1966; Raven, 1966; Hyman, 1967; Kriegstein, 1977). The buccal ganglia arose in the first third of larval life in *Ilyanassa,* well after feeding had beg-un, as happens in *Strombus gigas* (D'Asaro, 1965) and the non-feeding larvae of *Patella*

vulgata (Smith, 1935). The relatively late arrival of the buccal ganglia suggests that the buccals have little functional significance for the control of larval feeding activities.

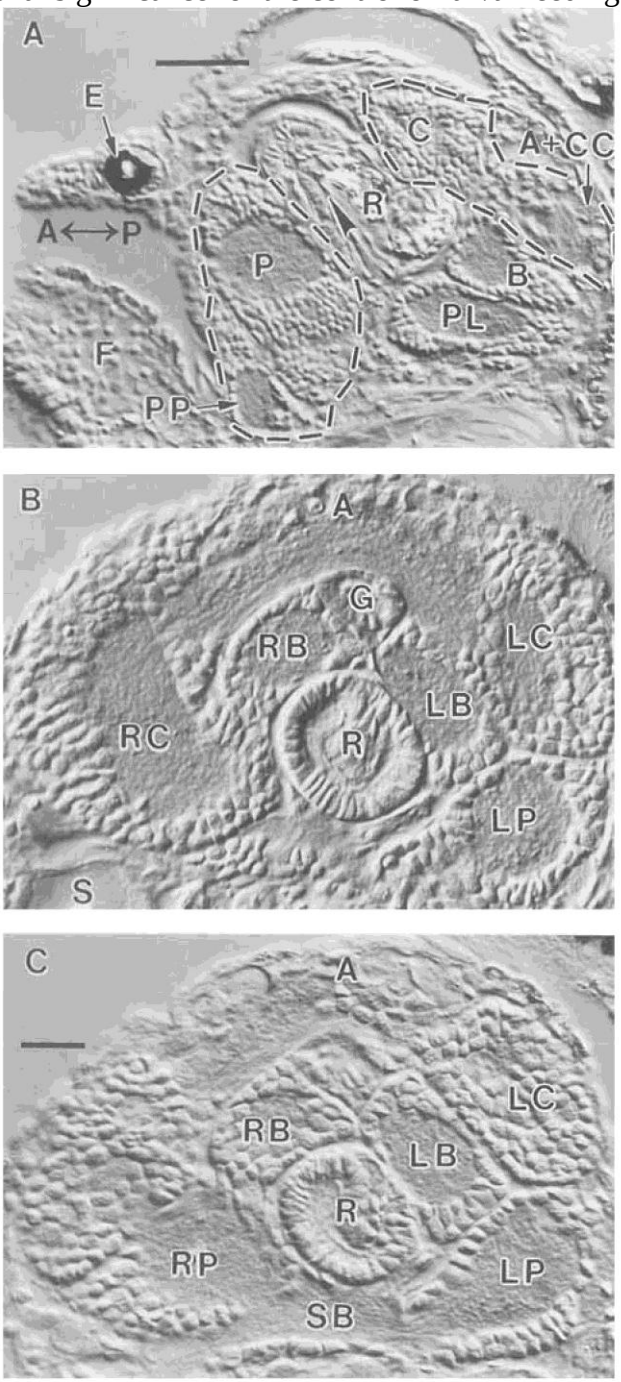

Fig. 12. Sagittal A and transverse B,C, sections through newly metamorphosed juveniles. **A–C:** The apical ganglion and the cerebral commissure are now located in the posterior part of the head, just above the buccal ganglia. The buccal ganglia of newly metamorphosed juveniles are medial to the cerebral ganglia and are closer to each other than in competent larvae. The buccal commissure is not visible at this magnification. Note that the radular sac has moved upwards (arrowhead in A). (A+CC, apical ganglion and cerebral commissure.) **C:** Note continuity of neuropil of subintestinal with those of both pleural ganglia. Scale bars = 50 μm in A, 20 μm in C.

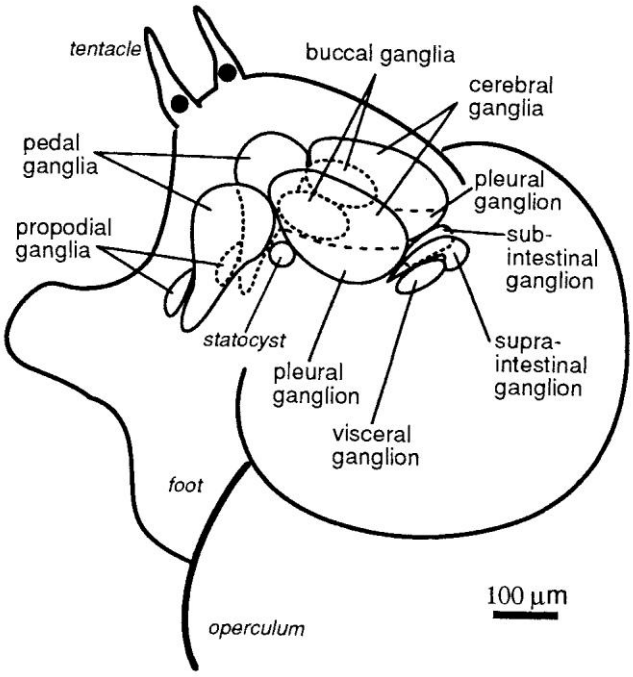

Fig. 13. Diagram of an advanced juvenile showing the relationship of the ganglia of the CNS. There is no apical ganglion. The cerebral ganglia are now caudal to the pedals and the pleurals remain fused with the cerebrals in back of the statocysts to form the cerebropleural ganglia. The subintestinal ganglion is mostly posterior to the right cerebropleural ganglion, but its neuropil is connected to both pleural ganglia (cf. Fig. 12C). The supraintestinal ganglion curves obliquely above the subintestinal and visceral ganglia. Propodial ganglia occur near the anteroventral region of each pedal ganglion.

The intestinal ganglia arose between 8 and 12 days after hatching, midway through the veliger stage, although they arise relatively later in other prosobranch veligers (D'Asaro, 1965, 1966, 1969; Bedford, 1966). This variability in the ontogeny of these ganglia supports Page's (1994) assertion of the commonality of heterochrony in the advanced gastropods. In larval *Ilyanassa*, the neuropils of both intestinal ganglia were fused with that of the right pleural ganglion. After metamorphosis the neuropil of the subintestinal ganglion only was fused with that of both pleurals. This is similar to the situation described in other caenogastropods. In *Crepidula adunca*

(Moritz, 1939), *Marisa cornuarietis* (Demian and Yousif, 1975), and *Ampullaris sp.* (Honegger, 1974), the subintestinal ganglion also appears to be fused with the right pleural ganglion, while the supraintestinal comes to lie near the left pleural ganglion, as it did in *Ilyanassa.* In *Ilyanassa,* these close ganglionic connections remain in the adults (Bouvier, 1887). The visceral ganglion became distinctive only during competence in *Ilyanassa* larvae. Elements of the visceral ganglion could be present earlier, but may only be evident under the electron micro-scope (Page, 1994). In juveniles, the neuropil of this ganglion was fused with that of the subintestinal. The adult configuration, which includes the division of the visceral ganglion into two distinct ganglia, the separation of the viscerals from the intestinal ganglia on long connectives, and a visceral-supraintestinal connective (Bouvier, 1887), must arise well after metamorphosis.

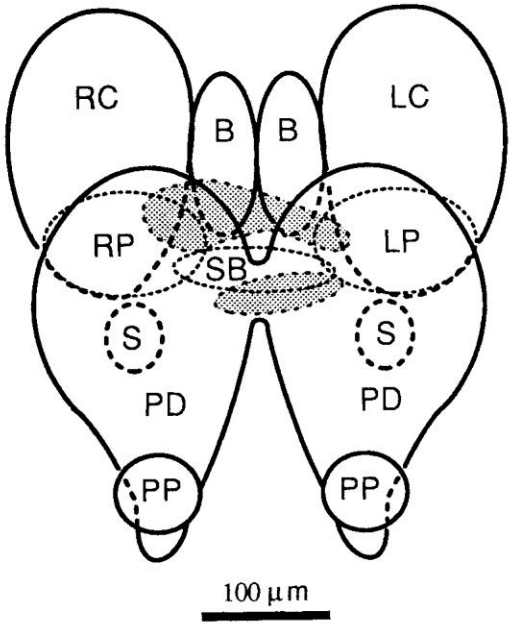

Fig. 14. Relationship of the major ganglia of an advanced juvenile viewed transversely looking towards the posterior end of the animal. The buccal ganglia are internal to the cerebrals and larger than those of competent larvae. The pleural ganglia occur at the caudal end of the cerebral ganglia, above the statocysts. The largest diameters of the cerebropleural ganglia are not shown here. Ganglia of the visceral loop occur behind the fused cerebropleural ganglia. Two of the most posterior ganglia, the supraintestinal (upper) and visceral (lower), are stippled and unlabeled.

The organs involved in feeding are drastically reorganized at metamorphosis in both prosobranchs and opisthobranchs (D'Asaro, 1965; Fretter, 1969; Kriegstein et al., 1974; Bickell et al., 1981; Marois and Carew, 1990). In *Ilyanassa,* the buccal mass was well developed and the radula present at metamorphosis, but the feeding response was not inducible until 4 days after metamorphic induction. A similar cessation of feeding during metamorphosis has been described in opisthobranch larvae. Movement of the buccal mass was recorded by Switzer-Dunlap and Hadfield (1977) on the second day after metamorphosis in *Aplysia juliana* and on the third day after the induction of metamorphosis in *Aplysia californica* (Kriegstein et al., 1974). Furthermore, the food preferences of newly metamorphosed juveniles often appear to be similar to those of competent larvae. For example, newly metamorphosed juveniles of *Onehidoris bilamellata* are incapable of feeding on adult food items and mostly ingest detritus (Chia and Koss, 1989). Similarly, juvenile *Ilyanassa* tend to be obligate herbivores that grow well on monocultures of benthic diatoms (Brenchley, 1987). Brenchley did not study newly metamorphosed juveniles, but such animals also appear to be herbivores, ingesting diatoms and giving no response to fish carrion that is strongly chemoattractive to adult *Ilyanassa.*

The translocation of the feeding organs is profoundly interrelated to the location of the CNS during and after metamorphosis. In *Ilyanassa* larvae, the radular sac developed beneath the esophagus, was dorsal to the statocysts, and lay between the fused cerebropleural ganglia. After metamorphosis, the buccal mass, within the developing proboscis, came to lie completely above the ganglia of the CNS. The larval radular sac had no opening to the exterior, but when the velum was cast off, the anterior end of the proboscis sheath opened to join

the anterior end of the larval gut (Fretter, 1969; Lin, personal observation). In *Haliotis* (Barlow and Truman, 1992) and *Ilyanassa*, as metamorphosis proceeds, the buccal musculature enlarges and is correlated with a movement of the cerebral ganglia to more posterior and lateral positions.

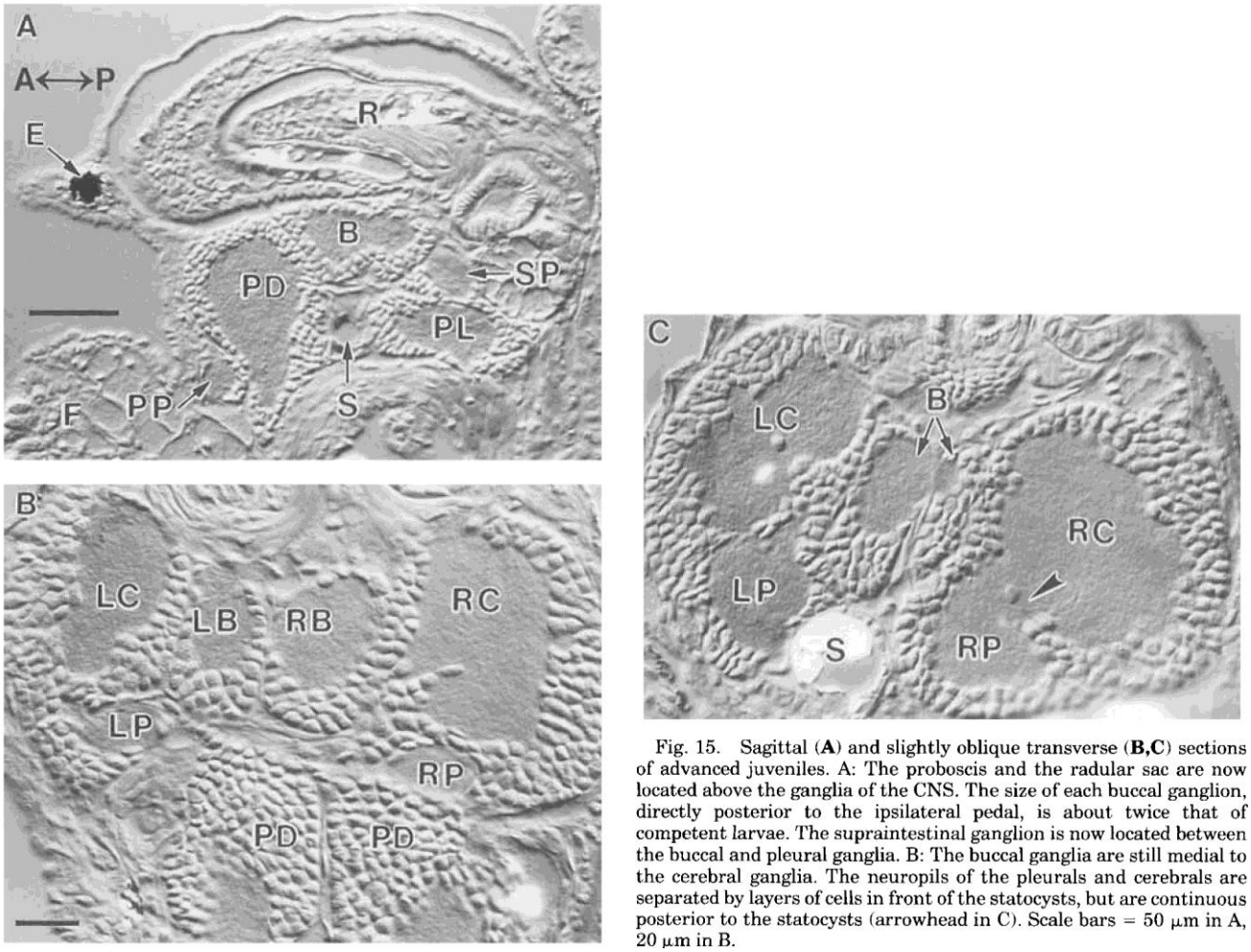

Fig. 15. Sagittal (**A**) and slightly oblique transverse (**B,C**) sections of advanced juveniles. A: The proboscis and the radular sac are now located above the ganglia of the CNS. The size of each buccal ganglion, directly posterior to the ipsilateral pedal, is about twice that of competent larvae. The supraintestinal ganglion is now located between the buccal and pleural ganglia. B: The buccal ganglia are still medial to the cerebral ganglia. The neuropils of the pleurals and cerebrals are separated by layers of cells in front of the statocysts, but are continuous posterior to the statocysts (arrowhead in C). Scale bars = 50 μm in A, 20 μm in B.

In many caenogastropods, water enters the mantle cavity via the siphon, which can be moved through the water column for selective sampling. Water then passes over the osphradium before being ejected from the mantle cavity. The osphradium is thus in a position to detect chemical gradients (Croll, 1983; Laverack, 1988; Emery, 1992). In *Ilyanassa* veligers, the osphradium and its subepidermal ganglion were distinct by 6 days after hatching. Its potential role in larval behaviors, such as the chemosensory initiation of metamorphosis, selective feeding, or detection of conspecifics, remains to be determined.

In *Ilyanassa* (Lin and Leise, 1994; Leise, 1996) and in the sea hare *Aplysia californica* (Marois et al., 1993), a distinct apical ganglion has been identified that lies above the dorsal cerebral commissure. This apical ganglion is most likely a developmental outgrowth of the trochophore apical plate (Raven, 1966) and an evolutionary enlargement of an apical or cephalic sensory organ like the ones described in other gastropods (Conklin, 1897; Fretter and Graham, 1962; D'Asaro, 1969; Bonar, 1978a,b; Morse et al., 1980; Bickell and Kempf, 1983; Chia and Koss, 1984; Marois and Carew, 1990; Kempf et al., 1991; Page, 1992a, 1993, 1994). Both *Aplysia* and *Ilyanassa* have relatively long-lived planktotrophic veligers that are large at metamorphic competence in comparison to the non-feeding larvae of many of their opisthobranch relatives. Further comparative studies and a better understanding of this ganglion's functions are needed to determine if the possession of a distinctive apical ganglion is a necessary foundation for a relatively long planktotrophic life history phase.

In *Phestilla sibogae* (Bonar, 1978a,b) and *Rostanga pulchra* (Chia and Koss, 1984), the apical sensory organ contains neurons with superficial dendrites and axons that penetrate the underlying cerebral commissure. The function of these putative chemosensory neurons is still speculative, but their most likely task is to mediate the

chemosensory induction of metamorphosis (Bonar, 1978a). In *Aplysia californica*, *Berghia verrucicornis*, and *Ilyanassa obsoleta*, the apical ganglion contains several serotonergic neurons that innervate the velum (Marois et al, 1993; Kempf et al., 1991 and Kempf, personal communication). Serotonin is active in the metamorphic pathway in *Ilyanassa*, as injected serotonin (5-HT), the re-uptake inhibitor fluoxetine, and the serotonergic agonist α-methyl-5-HT, all induce metamorphosis (Couper and Leise, 1996). However, we do not yet know if these actions are mimicking the activity of the serotonergic neurons in the apical ganglion. Some of the serotonergic neurons in the apical ganglion of *Aplysia* have processes that extend to the surface of the animal (R. Marois, personal communication), suggesting that they may have sensory functions. However, chemosensory receptors that might mediate larval settlement and the induction of metamorphosis can be located in other larval structures, such as the anterior (Chia and Koss, 1989; Arkett et al., 1989) or posterior (D'Asaro, 1966) ends of the foot.

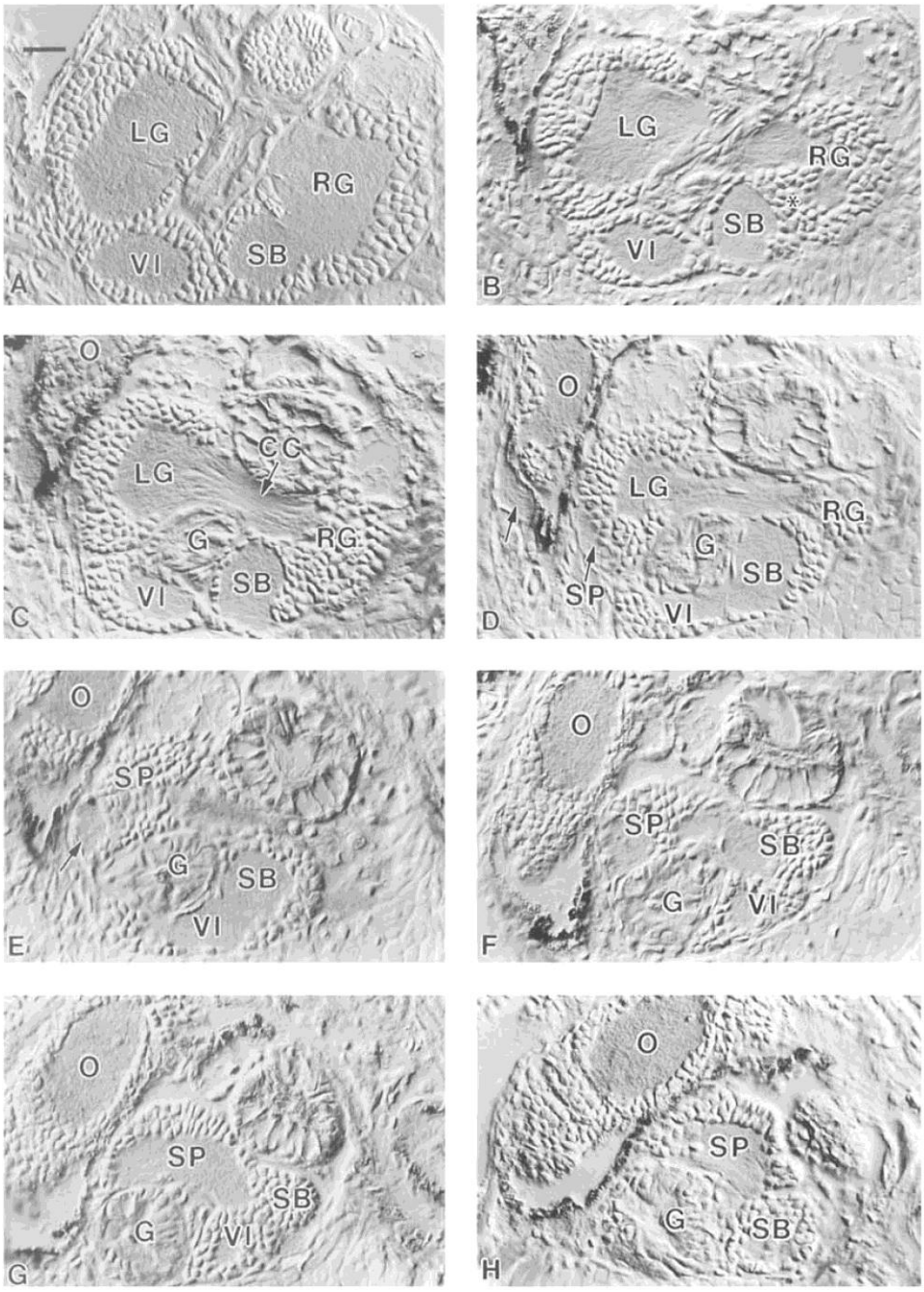

Fig. 16. Transverse serial sections from an advanced juvenile showing the relationship of the intestinal and visceral ganglia. **A:** The visceral ganglion is ovoid in shape, lies below the caudal portion of the left cerebropleural ganglion, and extends into the right half of the animal. The neuropils of the subintestinal and the right cerebropleural ganglia are fused. **B:** More posteriorly, the neuropils of the subintestinal and right cerebropleural ganglion are separated by several layers of somata (asterisk). **C:** A commissure now connects the caudal region of the cerebropleural ganglia. **D,E:** The neuropils of the subintestinal and right portion of the visceral ganglion are fused. A nerve (arrows) from the supraintestinal ganglion leads to the osphradium. **F-H:** The supraintestinal ganglion curves above the subintestinal and visceral ganglia and the neuropil of the supraintestinal ganglion is fused with that of the subintestinal ganglion. The caudal end of the visceral ganglion is shifted obliquely to the right side of the animal. Scale bar = 20 μm.

TABLE 1. Ontogeny of the Ganglia in Larval *Ilyanassa obsoleta*

6 Days after hatching	8 Days after hatching	12 Days after hatching	Competent larva
Apical (A)	A	A	A
Osphradial (O)	O	O	O
Cerebral (C)	C	C	C
Pedal (PD)	PD	PD	PD
	Pleural (PL)	PL	PL
	Buccal (B)	B	B
	Supraintestinal (SP)	SP	SP
	Subintestinal (SB)	SB	SB
			Visceral
			Propodial

In *Phestilla sibogae* (Bonar, 1978b) and *Ilyanassa*, the apical organ or ganglion persists through metamorphosis into 2-day-old juveniles; its ultimate metamorphic fate is unknown. The early ontogenetic appearance of this ganglion, followed by its subsequent reorganization and then disappearance in advanced juveniles, suggests that its functions are critical to larval behaviors and that it could even be active during metamorphosis, becoming non-functional as the juveniles mature. The apical ganglion appears to be the only significant neural structure that is lost during prosobranch metamorphosis. This small collection of neurons undoubtedly holds the key to our under-standing the neuronal mechanisms that control diverse aspects of larval molluscan behavior.

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